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Original Article—

Inactivation of Three Subtypes of Influenza A Virus by a Commercial Device Using Ultraviolet Light and Ozone

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SUMMARY. Avian influenza (AI) is a highly contagious disease that can be transmitted to naïve birds through fomites. The survival of AI viruses (AIV) on nonporous and porous fomites also dictates how long the fomite can serve as a vehicle for virus transmission. AIVs are known to be inactivated by ozone and ultraviolet (UV) light. However, the combined effect of UV light and ozone in combating AIV on different fomites has not been investigated. This study was undertaken to determine AIV inactivation by a commercial device called the BioSec shoe sanitizing station. This device generates both ozone and UV light for 8 sec when activated. We evaluated this device against three different subtypes of AIVs applied on seven different fomites. In general, the device inactivated all three AIV subtypes loaded on all fomites but to varying degrees of inactivation. The percentage of virus reduction on nonporous fomites (98.6%–99.9%) was higher than on porous fomites (90.0%–99.5%). In conclusion, this new device has the potential to help reduce the risk of transmission of AIV.

RESUMEN. Inactivación de cuatro subtipos del virus de la influenza A mediante un dispositivo comercial usando luz ultravioleta y ozono.

La influenza aviar (IA) es una enfermedad altamente contagiosa que puede transmitirse a aves susceptibles a través de fómites. La supervivencia de los virus de la influenza aviar en fómites porosos y no porosos también determina cuánto tiempo el fómite puede servir como vehículo para la transmisión del virus. Se sabe que los virus de influenza aviar son inactivados por el ozono y la luz ultravioleta (UV). Sin embargo, no se ha investigado el efecto combinado de la luz ultravioleta y el ozono para inactivar el virus de la influenza aviar en diferentes fómites. Este estudio se llevó a cabo para determinar la inactivación del virus de la influenza aviar mediante un dispositivo comercial llamado estación de desinfección de calzado BioSec. Este dispositivo genera ozono y luz ultravioleta durante 8 segundos cuando se activa. Se evaluó este dispositivo frente a cuatro subtipos diferentes del virus influenza aviar aplicados en siete fómites diferentes. En general, el dispositivo inactivó los cuatro subtipos de influenza aviar inoculados en todos los fómites, pero con distintos grados de inactivación. El porcentaje de reducción de virus en fómites no porosos (98.6%–99.9%) fue mayor que en fómites porosos (90.0%–99.5%). En conclusión, este nuevo dispositivo tiene el potencial de ayudar a reducir el riesgo de transmisión del virus de la influenza aviar.

Key words: influenza, ozone, ultraviolet, fomites

Abbreviations: AIV = avian influenza virus; AL = aluminum; BSA = bovine serum albumin; CB = cardboard; DMEM = Dulbecco's Modified Eagle Medium; FB = denim fabric; HPAIV = highly pathogenic avian influenza virus; LPAIV = low pathogenic avian influenza virus; MDCK = Madin-Darby canine kidney; PP = polypropylene; RB = rubber boots; SS = stainless steel; SSS = BioSec shoe sanitizing station; UV = ultraviolet; ST = Styrofoam

Avian influenza virus (AIV) is an enveloped RNA virus belonging to the family *Orthomyxoviridae*. The virus causes respiratory infection in domestic poultry and is divided into highly pathogenic avian influenza virus (HPAIV) and low pathogenic avian influenza virus (LPAIV) according to their potential to cause severe or mild disease, respectively (1). However, some LPAIVs can mutate to become more pathogenic and cause severe illness (2). The AIVs are divided into antigenic subtypes according to their surface glycoproteins, known as hemagglutinin (H) and neuraminidase (N). Currently, 18 hemagglutinin subtypes (H1–H18) and 11 neuraminidase subtypes (N1–N11) are recognized. Of these, only H1–H16 and N1–N9 subtypes are enzootic in avian species (3, 4).

The AIV is transmissible to susceptible hosts either directly by inhalation or indirectly through contact with contaminated inanimate objects (e.g., fomites). Fomites often play a crucial role in introducing AIV onto animal and poultry farms. The survival of viruses on nonporous and porous fomites also dictates how long the fomite can serve as a vehicle for virus transmission (5,6).

The AIVs are sensitive to various chemical disinfectants, ozone, and ultraviolet (UV) light. The UV light, also known as electromagnetic radiation, differs from visible light because of its shorter wavelength. There are three known categories of UV light according to their wavelengths: UV-A (320–400 nm), UV-B (290–320 nm), and UV-C (100–290 nm). The latter is the most powerful with the highest energy (7). Technologies using UV light have helped combat human and animal pathogens (8). It should also be noted that UV light with short wavelength (below 240 nm) can also generate ozone (with the chemical formula O_3), which is a powerful disinfectant and is characterized by a distinctive smell.

During 2015 there were sudden outbreaks of AIV H5N2, H5N8, and H5N1 subtypes in 15 states of the United States resulting in a high mortality rate among infected flocks. This, along with forced depopulation of infected farms, led to significant

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Fig. 1. Activation of BioSec SSS by standing on one screen while fomites (red arrow) are placed upside down on the second screen.

economic losses estimated to be over US$3 billion (9). This current study was aimed to investigate the combined effect of UV and ozone in combating three different LPAIV subtypes applied to seven different fomites. The hypothesis was that all subtypes will behave the same way on different fomites when exposed to ozone and UV light.

MATERIALS AND METHODS

Viruses. Three strains of LPAIV (H4N8 [A/mallard/Minnesota/Sg-00070/2007], H4N6 [A/mallard/Minnesota/Sg-00045/2007], and H9N9 [A/mallard/Minnesota/Sg-00245/2008]) were grown and titrated in Madin-Darby canine kidney (MDCK) cells. The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) containing 8% fetal bovine serum, 1 µg/ml fungizone, 50 µg/ml neomycin, 150 IU/ml penicillin, and 150 µg/ml streptomycin.

Source of UV and ozone. A commercial device called the "BioSec shoe sanitizing station (SSS)" (Fig. 1) was obtained from Pathogen Solutions (St. Petersburg, FL). This machine produces ozone (6.0 ppm) at a wavelength of 185 nm and UV-C (2000 µW/cm^2) at a wavelength of 254 nm. The machine has four UV (386 mm) and two ozone (357 mm) lamps and is certified by a Nationally Recognized Test Laboratory for safe use. The machine sterilization cycle, which lasts a total of 8 sec, begins automatically when someone stands on the glass shield of the machine (Fig. 1).

Procedure. All experiments were conducted at room temperature (~25 C) with ~50% relative humidity. Seven different fomites were tested, namely aluminum (AL), rubber boots (RB), cardboard (CB), denim fabric (FB), polypropylene (PP), stainless steel (SS), and Styrofoam (ST). Using sterile forceps, circular pieces of fomites (~1 cm^2) were placed in two 24-well tissue culture plates (one each serving as treated and untreated plate) within a biological safety cabinet. All fomites were loaded with 60 µl of a given virus per fomite. The virus was loaded by a micropipette and was spread with the help of this micropipette. Both plates were left open for 8 hr in a biological safety cabinet to dry so that the virus did not drip when placed upside down on the SSS. Using sterile forceps, fomites of untreated control plate were placed upside down on one screen of the shoe sanitizer machine for 8 sec but without starting the sterilization cycle. These fomites were then collected in a new 24-well tissue culture plate. The same procedure was used with the treated plate except that the fomites from this plate were placed on the left-side screen of the SSS and the machine was activated/started by standing on the right-side screen (Fig. 1). A beeping sound indicated the end of the 8-sec cycle. The treated fomites were also collected in another new 24-well tissue culture plate.

The surviving viruses were eluted from the fomites using 100 µl of elution buffer (3% beef extract in 0.05 M glycine) per fomite. Serial 10-fold dilutions of all eluates were prepared in maintenance medium consisting of DMEM, 0.45 µg/ml of tosyl phenylalanyl chloromethyl ketone–treated trypsin, 2% bovine serum albumin (BSA), 1 µg/ml fungizone, 50 µg/ml neomycin, 150 IU/ml penicillin, and 150 µg/ml streptomycin. All dilutions were inoculated in monolayers of MDCK cells contained in 96-well plates using three wells per dilution. The plates were incubated at 37 C under 5% CO_2 and examined daily for 7 days for the appearance of virus-specific cytopathic effects. Virus titers were calculated using the Karber method (10). The experiment was repeated once, and the amount of average virus reduction was calculated by subtracting virus titer in "treated" fomites with those in the "control" fomites.

RESULTS

Viability reduction of H9N9 on RB was 93% while reduction of H4N8 and H4N6 was 99% and 98.5%, respectively. Reduction of all three strains on CB was low (78.6%). Reduction of these viruses on FB was between 90% and 98.5%. On PP, the reductions were between 98.9% and 99.9%. The percentage of reduction of all three viruses on SS discs ranged from 99.5% to 99.98%. The inactivation rate for H4N8, H4N6, and H9N9 loaded on PP was between 99.7%

Table 1. Inactivation of AIV subtypes on different fomites.

Virus strain	Sample Type	AL	RB	CB	FB	PP	SS	ST
H4N8	Control (log $TCID_{50}$/0.1 ml)[AB]	3.9	3.83	2.5	4.5	4.16	3.9	4.33
	Treated (log $TCID_{50}$/0.1 ml)	2.0	1.83	1.83	2.7	0.4	0	2.83
	Reduction (%)	98.9	99.0	78.6	98.5	99.98	99.98	96.8
H4N6	Control (log $TCID_{50}$/0.1 ml)	3.83	4.0	1.83	3.83	4.2	3.9	3.16
	Treated (log $TCID_{50}$/0.1 ml)	1.9	2.16	1.16	2.83	1.6	1.66	1.5
	Reduction (%)	98.6	98.5	78.6	96.4	99.7	99.5	97.8
H9N9	Control (log $TCID_{50}$/0.1 ml)	4.33	4.5	2.83	4.5	4.33	4.5	3.83
	Treated (log $TCID_{50}$/0.1 ml)	2.5	3.33	2.16	2.9	1.5	2.0	2.16
	Reduction (%)	98.6	93.2	78.6	96.9	99.8	99.6	97.8

[A]$TCID_{50}$ = 50% tissue culture infectious dose.
[B]Numbers represent average virus titer.

and 99.9%. Reduction rate for H4N6 and H9N9 on ST was 97.8% while reduction in H4N8 titer was 96.8% (Table 1; Fig. 2). In general, the machine inactivated all AIV strains loaded on all fomites. The reduction percentage on nonporous fomites (98.6%–99.9%) was higher than on porous fomites (e.g., ST [96.8%–99.5%], FB [90.0%–98.5%], and RB [93.2%–99.0%]).

DISCUSSION

Several types of fomites are present on the farm including drinkers, feeders, cages, apparel, and egg trays. These fomites become contaminated with viral pathogens once animals are actively shedding. Inactivation of viruses on various fomites is, therefore, a crucial biosecurity practice to combat the adverse effects of pathogens. The survival of AIV on various fomites has been reported. For example, Tiwari *et al.* (5) observed that AIV could survive for 3 to 9 days on most fomites while an HPAI subtype was found to persist for more than 13 days on glass and steel (11).

UV light and ozone have separately been shown to inactivate a wide range of microorganisms in various milieu, e.g., food, water, air, equipment, and surfaces (12, 13, 14). However, no study is available assessing the combined effect of these two technologies on AIV inactivation although Novak *et al.* (15) suggested that ozone as a strong oxidizer should have a synergistic effect with UV light because the latter has lower capacity of invasion and diffusion.

The LPAIVs were used in this study as a surrogate for HPAIVs (16). Because of the effect of temperature, humidity, and oxygen concentration on the generated ozone, all factors were kept constant throughout the experiment (17). The combination of UV light and ozone inactivated all AIV strains loaded on porous and nonporous fomites. The variation in virus reduction among different subtypes can partly be due to escape of the viruses between fibers or pores of porous fomites.

It should be mentioned that these experiments were conducted with viruses dried on various fomites. How these viruses behave when suspended in liquids is not known and should be investigated

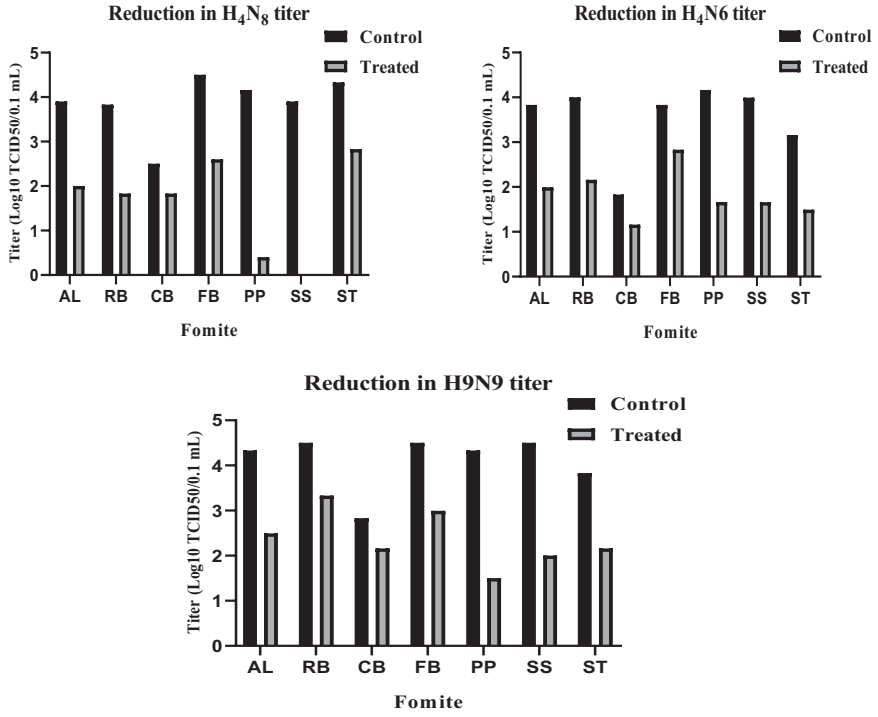

Fig. 2. Reduction in AIV titers after loading on different fomites and exposing to the BioSec SSS for eight seconds.

(18). Different fomites may react with UV light and ozone differently. Several studies are available on the inactivation of AIVs by UV light. For example, Shahid *et al.* (19) found that 50% of AIV was killed after 60 min of exposure to UV light. Similarly, Muhammad *et al.* (20) reported that UV light was not sufficient for absolute inactivation of H7N3 strain after 45 min of exposure.

McDevitt *et al.* (21) found that influenza virus in aerosol was susceptible to UV-C and that the virucidal efficacy of UV light increased with decreasing relative humidity. This can partially explain an increased number of influenza epidemics in winter because low amounts of UV light can reach the ground due to clouds and/or pollution (22). The UV efficiency was found to inactivate 99.99% AIV in virus aerosols (23). Similarly, 10 ppm of ozone inactivated 99.99% of AIV on plastic after 210 min. At a higher concentration (i.e., 20 ppm), ozone killed 99.99% of the virus within 150 min (24). The current study was designed to determine the combined effect of UV light and ozone on AIV.

All AIVs are susceptible to UV radiation because these viruses contain a lipoprotein envelope that is weaker than nonenveloped DNA viruses; for example, nonenveloped adenovirus can resist 254 nm UV-C (25). No correlation has been found between response to UV radiation and genetic composition or virion size of a virus (26). Hence, different genetic compositions of the tested viruses may not be the cause of variation in the reduction ratio between different strains after treatment with ozone and UV light. Slight variation in virus inactivation may have been caused because of some study limitations. One limitation is that we loaded fomite with a virus that may stack as one droplet with virus clumping or randomly spread on the fomite surface. Another limitation is that the fomites were distributed randomly on the SSS glass shield which may interfere with the distribution of emitted rays and subsequently influence sterilization power. Further studies to determine the exact UV and ozone dose to kill AIV on different fomites are needed.

These experiments were performed without any soil or organic matter overload, which may need more exposure time of scattered light to get the same reduction rate. Viruses can escape the effect of UV radiation by being embedded in contaminants due to the canyon-wall effect (27). Kaoud *et al.* (28) observed that UV light is not useful in killing AIV in unsolid fecal material. Accordingly, we believe that the optimum reduction of AIVs by the SSS can be obtained if shoes are first cleaned of mud or dust to reduce contaminant overload.

Characterizing the effectiveness of the UV-C and ozone combination on AIV after exposure for a fixed period (8 sec) on different fomites provides a scientific basis for styling a new and more effective generation of devices. The combination of ozone and UV light can be used for sterilization of trucks, clothes, towel sterilization cabinets, adaptors in air outlets and inlets, and UV rooms; entering and exiting materials can be processed on devices that may look like baggage scanner machines. We hope this work attracts the attention of poultry producers including hatcheries, farms, the feed industry, farming-supply owners, and production distributors for broader implementation of UV-C radiation, and its accompanied generated ozone as a mitigation procedure for AIV. In conclusion, the availability of combined UV and ozone technology should help limit the adverse effect of an AIV outbreak.

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